Production of Aspergillus niger GH1 Tannase using Solid-State Fermentation

Mario Cruz-Hernández¹, Juan Carlos Contreras¹, Nelson Lima⁴, Jose Teixeira⁴ and Cristobal Aguilar¹*¹

¹Food Research Department, Universidad Autónoma de Coahuila, 25000 Saltillo, Coahuila, México.
²IBB - Institute for Biotechnology and Bioengineering, Centre for Biological Engineering, Universidade do Minho, Campus de Gualtar, 4710-057 Braga, Portugal.

(Received: 15 October 2008; accepted: 21 December 2008)

The production of tannase by Aspergillus niger GH1 in solid-state fermentation, under different initial concentrations of tannic acid (12.5, 25, 50 and 100 g l⁻¹). The reactors were packed with polyurethane foam impregnated with medium and inoculated with fungal spores. Tannase production was kinetically monitored by 48 h. The tannase activities extracellular and intracellular were assayed by HPLC-chromatography. Maximum extracellular and intracellular tannase activities (11.35 and 6.95 U ml⁻¹ respectively) were recorded with 100 g l⁻¹ of tannic acid. The substrate uptake was 100% at concentrations of 12.5, 25 and 50 g l⁻¹, while 74.4% was consumed in the presence of 100 g l⁻¹ of tannic acid after 48 h of culture. These results suggest that high concentrations of tannins can be removed and tannase production can be reached in high levels. The needed to establish the production conditions under solid state fermentation, a system where the tannase is expressed extracellular in high levels.

Key words: Tannase, Tannic acid, Solid-state fermentation, Aspergillus niger GH1.

Tannins are molecules considered as water-soluble phenolic compounds with molecular weights ranging from 500 to 3000 Da. Tannins are present in several plants acting as pigments¹ and protecting agents against microbial attacks². Several proposals to use the tannin-rich plants as a source of added value products have been made. One of them, and the most frequently one, is related with its use as substrate for the production of microbial enzymes, in particular tannase³.

Tannin acyl hydrolase (E.C. 3.1.1.20), commonly called tannase, is an important enzyme used industrially for the manufacture of gallic acid from tannin-rich materials. The tannase catalyzes the hydrolysis of ester and depside bonds in such hydrolysable tannins as tannic acid. It is generally used in food and beverage processing⁴, namely, in the production of instant tea, coffee flavoured soft drinks and in the clarification of beer and fruit juices⁵.

* To whom all correspondence should be addressed.
Fax: + 52 (844) 4161238
e-mail: cag13761@mail.uadec.mx
Production of tannase by various bacterial\textsuperscript{7} and fungal strains\textsuperscript{8-11} has been reported. Different works reported liquid surface, submerged or solid-state fermentation for tannase production. Among these, submerged fermentation process is mostly preferred because the sterilization and process-control methods are easier in this system\textsuperscript{3}. Although vast amount of tannase production was achieved by submerged fermentation. This method implies some advantages mainly in the production cost and the instability of the produced enzyme\textsuperscript{6,12}. In this aspect, production of such enzyme from fungal strains through solid-state fermentation (SSF) is cheaper, less technology is required and its extraction is easier\textsuperscript{13}. An important advantage of SSF is that it produces only negligible amount of liquid effluents and thereby creates less pollution\textsuperscript{14}. According our knowledge, only few reports on tannase production through SSF by \textit{Aspergillus niger}\textsuperscript{15,16,17}, \textit{Aspergillus aculeatus}\textsuperscript{18}, \textit{Paecilomyces variotii}\textsuperscript{19}, \textit{Aspergillus foetidus} and \textit{Rhizopus oryzae}\textsuperscript{20} are available. Tannase production through liquid submerged fermentation and SSF by \textit{Aspergillus niger} GH1 was reported earlier\textsuperscript{9} and the potential of using agro-industrial wastes as substrate for industrial tannase production through SSF has been considered. In this work, fermentation parameters for tannase production and tannic acid degradation by \textit{Aspergillus niger} GH1 in a model SSF system were evaluated at different tannic acid concentrations.

**MATERIALS AND METHODS**

**Microorganism and culture medium.** Spores of the strain \textit{Aspergillus niger} GH1 (DIA/UadeC collection) were preserved at 20°C in protect-crioblocks (bead storage system, Technical Service Consultants Limited). Inoculum was prepared transferring the spores to potato dextrose agar (PDA BD Bixon\textregistered), incubated at 30°C for 5 days. After the fungal growth the spores were scraped into 0.01% Tween 80 sterilized solution and counted in a Neubauer chamber. Medium for tannase production was the same as reported contained (g l\textsuperscript{-1}): KH\textsubscript{2}PO\textsubscript{4}, 5; NH\textsubscript{4}H\textsubscript{2}PO\textsubscript{4}, 10; MgSO\textsubscript{4}·7H\textsubscript{2}O, 1; CaCl\textsubscript{2}·6H\textsubscript{2}O, 0.1; MnCl\textsubscript{2}·6H\textsubscript{2}O, 0.02; NaMoO\textsubscript{4}·2H\textsubscript{2}O, 0.01; FeSO\textsubscript{4}·7H\textsubscript{2}O, 0.125. Salt-containing medium was autoclaved at 121°C for 15 min. Tannic acid (Sigma, U.S.A.) solution was filter-sterilized (nylon membrane 47 mm diameter, 0.2 μm pore size, Millipore) and added to a final concentration of 12.5, 25.0, 50.0 and 100.0 g l\textsuperscript{-1}.

**Solid state fermentation (SSF)**

The SSF involved the use of polyurethane foam (PUF) (Expomex, México) as a support to absorb the liquid medium. PUF was washed as reported (20) and then pulverised in a plastic-mill. Column reactors (25 × 180 mm) were packed with 10 g of inoculated (2 × 10\textsuperscript{6} spores g\textsuperscript{-1} of dry inert PUF) support. Culture conditions were: temperature 30°C, aeration rate 20 ml of air per gram of support per min, initial pH 5.5, and initial moisture content 65% and an incubation time of 48 h. Samples were taken every 6 h. At each sampling point, the fermented mass of reactor was removed and homogenized. For enzyme leaching, the content of each reactor was mixed with distilled water (10:1 w/v) and vortexed for 1 min. Solid particles were filtered (Whatman International Ltd. Springfield Mill, Maidstone, Kent, England, Filter No. 41) and the clear filtrate was assayed for extracellular tannase activity. The remaining solids were washed three times with 50 ml of distilled water. Intracellular enzyme was recovered by deep-freezing the cells in liquid nitrogen and by macerating in a chilled mortar. The recovery process was previously reported\textsuperscript{21}. The process was carried out using acetate buffer, pH 5.5 to recovery the enzyme from the debris.

**Analytical methods.** Tannase assay was carried out using the HPLC-methodology proposed\textsuperscript{22}. One unit of enzyme (U) was defined as the amount of enzyme able to release 1 μmol gallic acid per ml per min. Biomass formation in SSF was determined by technique reported\textsuperscript{23}, where 0.5 g of fermented solid is impregnated with phosphoric acid (0.15 mol l\textsuperscript{-1}) and heated in a boiling water bath to hydrolyse the mycelium during 7 min; the sample is then cooled and centrifuged to obtain a mycelial protein solution; finally, 200 ml of sample were mixed with 800 ml of biuret reagent and the blue color was measured at 595 nm. Tannic acid concentration was evaluated spectrophotometrically using the phenol-sulfuric acid method reported (Aguilar et al. 2001). Briefly, the method implies thermal reaction of 1 ml of sample with 2 ml of phenol-sulfuric reagent (1 mgml\textsuperscript{-1}) during 5 min into a boiling water bath.
then the sample was cooled and the absorbance
was recorded at 480 nm.

**Kinetics parameters**

Growth curves were fitted by a Maquardt
"Solver" computer program (Excel, Microsoft)
using logistic equation as follows:

\[ X = \frac{X_{\text{max}}}{1 + \left( \frac{X_{\text{max}} - X_0}{X_0} \right) e^{-\mu t}} \]  

Where \( X \) (gl\(^{-1}\)) represents the biomass
calculated, \( X_0 \) and \( X_{\text{max}} \) (gl\(^{-1}\)) are the initial and
maximum biomass value, respectively, \( \mu \) (h\(^{-1}\)) is
the specific growth rate, and \( t \) (h) is the culture
time. The algorithm minimizes the sum of least
square errors comparing experimental data with
the theoretical values obtained.

The biomass/substrate yield, \( Y_{x/s} \), is
calculated by the equation [2]:

\[ Y_{x/s} = \frac{(X_{\text{max}} - X_0)}{(S_0 - S_{\text{final}})} \]  

Where \( X_{\text{max}} \) and \( X_0 \) (gl\(^{-1}\)) are the maximum
and initial biomass values obtained, respectively,
and \( S_0 \) and \( S_{\text{final}} \) (gl\(^{-1}\)) are the initial and final
substrate concentration values, respectively.

The specific substrate uptake rate, \( q_s \), is
defined by the equation [3]:

\[ q_s = \frac{\mu}{Y_{x/s}} \]  

Where \( q_s \) is giving as grams of substrate
consumed per gram of biomass per hour.

Tannase/biomass yield, \( Y_{x/a} \), is estimated
from de linear correlation between tannase
activities, \( E \) (U\(^{-1}\)) and biomass concentrations, \( X \)
(gl\(^{-1}\)). The yield coefficient is defined as \( Y_{x/a} \) (units
of tannase per gram of \( X \)). The specific rate of
formation of enzymes, \( q_p \), is defined in [4]:

\[ q_p = \frac{\mu}{Y_{x/a}} \]  

Where \( q_p \) is the units of tannase produced
per gram of biomass per hour, \( Y_{x/a} \) is the tannase/
biomass yield.

**RESULTS AND DISCUSSION**

Both extracellular as intracellular tannase
activity produced by \( A. \) niger GH1 in the SSF
process were seriously affected by the initial tannic
acid concentration. Figure 1a shows the kinetics
of extracellular tannase production, while Figure 1b shows the results obtained for intracellular
tannase production. A consistent behaviour was
found, in which the increment in the initial tannic
acid concentration is related with an increase in
tannase activity. In this study, a maximum of
tannase activity was reached with 100 gl\(^{-1}\) of tannic
acid at 48 h. In other SSF systems evaluated, a
marked decrement in the tannase production after
to reach the maximum level is obtained However,
in this study that decrement was not found probably
due to the low level of concomitant protease
activity\(^{31}\). High levels of tannase produced were
excreted to the culture medium. Similar results
were obtained\(^{24}\) using sugar cane bagasse as
support of SSF demonstrating that the fungal
tannase activity was only detected in the
extracellular crude extract and not in the
intracellular extract.

This study revealed that the use of higher
tannic acid concentrations promotes the excretion
of tannase intracellular activity; these results may
be explained by the existence of a substrate
monitoring inside the cells that at high substrate
concentrations inhibits the tannase excretion
process. In this case, the extracellular/intracellular
ratio was 1.3:1 for 50 gl\(^{-1}\) and 1.6:1 for 100 gl\(^{-1}\); while for 12.5 and 25 gl\(^{-1}\) of tannic acid present,
the intracellular activities were low and the
extracellular/intracellular ratios were superior to
6:1 in both cases.

The results of tannase production
obtained in SSF system can be partially explained
by those reported for pectinesterase and
polygalacturonase\(^{25}\). These authors suggested that
the high levels of enzymatic production obtained
in SSF are due to changes in the composition of
membrane fatty acids provoked by stress
conditions favoured on this enzyme production
process (SSF) when the substrate concentration is
increased. Also, these results are explained by the
previous reports of our group\(^{13,17}\) where a different
pattern of induction/repression of \( \beta \)-galactosidase was
observed depending of the culture system and the

culture conditions. The most recent idea to explain the high levels of enzyme produced by SSF was proposed assuming the dependence of enzyme expression with the substrate and oxygen diffusivities on the solid support. However, this phenomenon has not been satisfactorily explained. Table 1 shows a summary of the kinetic parameters evaluated in SSF systems, being clearly demonstrated the high tannic acid-degrading capacity of \textit{A. niger} GH1. It is important point out that only at the highest substrate concentration there is no total consumption of tannic acid. This aspect is very important if the biodegradation of phenolic compounds or tannins of waste water and tannin-rich materials (i.e. coffee pulp, creosote bush, etc.) is to be considered.

Table 1. Summary of kinetic parameters considered in the tannase production and tannic acid degradation by SSF

<table>
<thead>
<tr>
<th>Tannic acid (gL⁻¹)</th>
<th>μ (h⁻¹)</th>
<th>$Y_{pX}$ (gXgS⁻¹)</th>
<th>$Y_{pX}$ (UmgX⁻¹)</th>
<th>$q_p$ (gXgS⁻¹h⁻¹)</th>
<th>$q_p$ (UgX⁻¹h⁻¹)</th>
<th>Substrate uptake (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.5</td>
<td>0.288</td>
<td>0.306</td>
<td>1.385</td>
<td>0.942</td>
<td>0.399</td>
<td>100</td>
</tr>
<tr>
<td>25.0</td>
<td>0.295</td>
<td>0.163</td>
<td>1.443</td>
<td>1.812</td>
<td>0.426</td>
<td>100</td>
</tr>
<tr>
<td>50.0</td>
<td>0.223</td>
<td>0.091</td>
<td>1.469</td>
<td>2.440</td>
<td>0.328</td>
<td>100</td>
</tr>
<tr>
<td>100.0</td>
<td>0.217</td>
<td>0.074</td>
<td>2.183</td>
<td>2.929</td>
<td>0.474</td>
<td>73.03</td>
</tr>
</tbody>
</table>

On SSF, a particular behaviour of the kinetic parameters is observed, as the product yield ($Y_p$), specific substrate uptake rate ($q_s$) and specific product formation rate ($q_p$) were higher for the initial tannic acid concentration of 100 gL⁻¹. Also, the higher whole tannase activity and lower uptake substrate percentage conversion were obtained. The analysis of the kinetic parameters demonstrated that SSF this is a good system to be applied in the degradation of hydrolysable tannins and tannase production.

Finally, it must be referred that several studies indicate that submerged fermentation (SmF) is not suitable for tannase production due to the long fermentation times (low productivity) and to the intracellular nature of the enzyme (2). The results presented in this work, using polyurethane foam as inert support, complement

![Fig. 1. Extracellular (a) and intracellular (b) tannase production by \textit{A. niger} GH1 in SSF with tannic acid concentrations of 12.5 (●), 25.0 (■), 50.0 (Δ) and 100.0 (+) gL⁻¹.](image)

those reported,\textsuperscript{15,19,18,12} and clearly demonstrate that SSF presents significant advantages as compared to submerged fermentation for tannase production, the fermentation time is improved, significant amounts of extracellular enzyme are produced and the system is not inhibited by high concentrations of tannic acid.

Obtained results clearly indicate the capability of \textit{Aspergillus niger} GH1 to produce high levels of tannase in SSF suggesting that this production system should be considered for large-scale production of tannase and gallic acid and that cheap and simple agricultural waste like coffee pulp or creosote bush may be used in the future as substrates.

\textbf{ACKNOWLEDGEMENTS}

The present work was performed as part of a cooperative agreement between the Universidad Autonoma de Coahuila (UAdEC, Mexico) and the Universidade do Minho (UM, Portugal) within a specific international exchange program (VALNATURA project, alfa network from European Union) undertaken at the Biological Engineering Department (UM, Portugal). M.A. Cruz-Hernandez thanks to Concejo nacional de Ciencia y Tecnología (CONACYT) the fellowship to study the doctoral program at the Department of Biotechnology (UAdEC).

\textbf{REFERENCES}


